MORAXELLA CATARRHALIS OUTER MEMBRANE PROTEIN-106 POLYPEPTIDE, GENE SEQUENCE AND USES THEREOF

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1. INTRODUCTION

The present invention generally relates to the outer membrane protein-106 (OMP106) polypeptide of *Moraxella catarrhalis*. The invention encompasses a purified OMP106 polypeptide and polypeptides derived therefrom (OMP106-derived polypeptides). The invention also encompasses antibodies, including cytotoxic antibodies, that specifically bind the OMP106 polypeptide and/or OMP106-derived polypeptides. The invention further encompasses prophylactic or therapeutic compositions, including vaccines, that comprise OMP106 polypeptide and/or OMP106-derived polypeptides. The invention additionally provides methods of inducing immune responses to *M. catarrhalis* in mammals. The invention further provides isolated nucleotide sequences encoding the OMP106 polypeptide and OMP106-derived polypeptides, vectors having said sequences, and host cells containing said vectors.

2. BACKGROUND OF THE INVENTION

Moraxella catarrhalis, also known as *Moraxella (Branhamella) catarrhalis or Branhamella catarrhalis* and formerly known as *Neisseria catarrhalis or Micrococcus catarrhalis*, is a gram-negative bacterium frequently found in the respiratory tract of humans. *M. catarrhalis*, originally thought to be a harmless commensal organism, is now recognized as an important pathogen in upper and lower respiratory tract infections in animals. In humans, *M. catarrhalis* causes serious lower respiratory tract infections in adults with chronic lung disease, systemic infections in immunocompromised patients, and otitis media and sinusitis in infants and children. See Helminen et al., 1993, Infect. Immun. 61:2003-2010; Catlin, B. W., 1990, Clin. Microbiol. Rev. 3:293-320; and references cited therein.

2.1. OUTER MEMBRANE PROTEINS AND PROTECTIVE ANTIBODIES

The outer surface components of *Moraxella catarrhalis* have been studied in attempts to understand the pathogenic process of *M. catarrhalis* infections and to develop useful therapeutic treatments and prophylactic measures against such infections. The outer membrane proteins (OMPs) in particular have received considerable attention as possible virulence factors and as potential vaccine antigens. *M. catarrhalis* has about 10 to 20 different OMPs with 6 to 8 of these, OMPs A to H, as the predominate species (Murphy and Loeb, 1989, Microbial Pathogen. 6:159-174). The molecular weights of OMPs A to H range from 97 to 20 kD, respectively. See Bartos and Murphy, 1988, J. Infect. Dis. 158:761-765; Helminen et al., 1993, Infect. Immun. 61:2003-2010; Murphy et al, 1993, Molecul. Microbiol. 10: 87-97; and Sarwar et al, 1992, Infect. Immun. 60:804-809. Comparisons of protein profiles by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane preparations from 50 *M. catarrhalis* strains show nearly homogeneous patterns of OMPs A to H (Bartos and Murphy, 1988, J. Infect. Dis. 158:761-765).

In addition to OMPs A to H, a high molecular weight OMP, designated HMW-OMP, having an apparent mass of 350 to 720 kD by SDS-PAGE has also been identified as another prominent surface component present in many strains of *M. catarrhalis*. HWM-OMP upon formic acid denaturation produces a single band of 120 to 140 kD and, thus, appears to be an oligomeric protein (Klingman and Murphy, 1994, Infect. Immun. 62:1150-1155). HMW-OMP appears to be the same protein as that designated UspA by Helminen et al., (1994, J. Infect. Dis. 170:867-872) and shown to be present in a number of *M. catarrhalis* strains.

In intact bacterium or bacterially-derived outer membrane vesicles, several of the above-identified OMPs present surface-exposed epitopes that elicit the production of antibodies that bind the OMPs. These antigenic OMPs

include OMP E and OMP G (Murphy and Bartos, 1989, Infect. Immun. 57:2938-2941); OMP C/D (Sarwar et al., 1992, Infect. Immun. 60:804-809); CopB, an 80 kD OMP, (Helminen et al., 1993, Infect. Immun. 61:2003-2010); and UspA (Helminen et al., 1994, J. Infect. Dis. 170:867-872).

The therapeutic potential of antibodies to surfaced-exposed epitopes of CopB and UspA has been evaluated in an animal model. The model involved direct bolus inoculation of lungs of BALB/c VAF/Plus mice with a controlled number of *M. catarrhalis* cells and subsequent examination of the rate of pulmonary clearance of the bacteria (Unhanand et al., 1992, J. Infect. Dis. 165:644-650). Different clinical isolates of the *M. catarrhalis* exhibited different rates of clearance that correlated with the level of granulocyte recruitment into the infection site. Passive immunization with a monoclonal antibody directed to a surface-exposed epitope of either CopB or UspA increased the rate of pulmonary clearance of *M. catarrhalis* (Helminen et al., 1993, Infect. Immun. 61:2003-2010; Helminen et al., 1994, J. Infect. Dis. 170:867-872).

2.2. BACTERIAL/HOST CELL ADHERENCE AND HEMAGGLUTINATION

The adherence of bacterial pathogens to a host cell surface promotes colonization and initiates pathogenesis. See, E.H. Beachey, 1981, J. Infect. Dis. 143:325-345. Gram-negative bacteria typically express surface lectins that bind to specific oligosaccharides of glycoproteins and/or glycolipids on the host cell surface. Such lectins are often associated with pili or fimbriae. Bacterial adherence can also occur by non-specific binding resulting from hydrophobic and/or charge interaction with the host cell surface.

The mechanism of *M. catarrhalis* adherence to cells of the respiratory tract remains poorly understood. The organism adheres to cultured human oropharyngeal epithelial cells (Mbaki et al., 1987, Tohuku J. Exp. Med. 153:111-121). A study by Rikitomi et al. suggests that fimbriae may have a role in the adherence to such cells as fimbriae denaturation

or treatment with anti-fimbriae antibodies reduced adherence by fimbriated strains (Rikitomi et al., 1991, Scand. J. Infect. Dis. 23:559-567). Fimbriae mediated binding, however, cannot be the sole basis of this adherence as the most highly adhering strain, among the several examined, was a non-fimbriated strain.

Hemagglutination reactions often replace the more complicated adherence assays in classifying bacterial adhesins. However, Rikitomi et al. found no correlation between human oropharyngeal epithelial cell adherence and hemagglutination by *M. catarrhalis* strains (Id.). That is three highly adhering strains did not agglutinate human erythrocytes. Thus, different binding mechanisms are involved in human oropharyngeal epithelial cell adherence and hemagglutination.

By contrast, a recent study by Kellens et al. suggests that hemagglutination by *M. catarrhalis* is correlated with host cell adherence (Kellens et al., 1995, Infection 23:37-41). However, this study employed an adherence assay based on bacterial binding to porcine tracheal sections. It is unclear whether porcine tracheal tissue can be considered homologous to human respiratory tract tissue with respect to adherence by pathogenic strains of *M. catarrhalis*.

Notwithstanding the problematic adherence assay, Kellens et al. examined the hemagglutination activities of eighty-some clinical isolates of *M. catarrhalis* (Kellens et al., 1995, Infection 23:37-41). Nearly three-quarters of the examined strains agglutinated human, rabbit, guinea pig, dog or rat erythrocytes, while the remaining strains did not. The agglutination activities for some of the hemagglutinating stains were further characterized and shown to be calcium ion dependent and inhibited by trypsin digestion or high-temperature treatment or addition of D-glucosamine or D-galactosamine.

A survey of hemagglutinating and non-hemagglutinating *M. catarrhalis* strains by Tucker et al. has

shown that all strains bind the glycolipid gangliotetraosylceramide but only hemagglutinating strains bind the glycolipid globotetraosylceramide (Tucker et al., 1994, Annual Meeting of Amer. Soc. Microbiol., Abstract No. D124). Moreover, *M. catarrhalis* hemagglutination activity was shown to be inhibited by various monosaccharides that comprise the carbohydrate moiety of globotetraosylceramide. These observations led Tucker et al. to suggest that *M. catarrhalis* hemagglutinates by binding to globotetraosylceramides in the cell membranes of susceptible erythrocytes, including those of human red blood cells. To date, no prior art has identified a molecule on the outer surface of *M. catarrhalis* that is responsible for either host cell adherence or hemagglutination.

Citation or identification of any reference in this section or any other section of this application shall not be construed as an indication that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention encompasses the OMP106 polypeptide of *M. catarrhalis* and OMP106-derived polypeptides and methods for making said polypeptides. The invention also encompasses antisera and antibodies, including cytotoxic antibodies, specific for the OMP106 polypeptide and/or OMP106-derived polypeptides. The invention further encompasses immunogenic, prophylactic or therapeutic compositions, including vaccines, comprising one or more of said polypeptides. The invention additionally encompasses nucleotide sequences encoding said polypeptides. The invention further encompasses immunogenic, prophylactic or therapeutic compositions, including vaccines, comprising an attentuated or inactivated non-hemagglutinating *M. catarrhalis* cultivar.

The present invention has many utilities. For example, the OMP106 polypeptide and OMP106-derived polypeptides may be used as ligands to detect antibodies

elicited in response to *M. catarrhalis* infections (e.g., in diagnosing *M. catarrhalis* infections). The OMP106 polypeptide and OMP106-derived polypeptides may also be used as immunogens for inducing *M. catarrhalis*-specific antibodies. Such antibodies are useful in immunoassays to detect *M. catarrhalis* in biological specimens. The cytotoxic antibodies of the invention are useful in passive immunizations against *M. catarrhalis* infections. The OMP106 polypeptide and OMP106-derived polypeptides may further be used as active ingredients in vaccines against *M. catarrhalis* infections.

The invention is based on the surprising discovery that hemagglutinating *M. catarrhalis* strains and cultivars have an outer membrane protein, OMP106 polypeptide, which is about 180 kD to about 230 kD in molecular weight, and that non-hemagglutinating *M. catarrhalis* strains and cultivars either do not have OMP106 polypeptide or have inappropriately-modified OMP106 polypeptide which is inactive in hemagglutination and not silver-stainable. The invention is further based on the discovery that polyclonal antiserum induced by OMP106 polypeptide isolated from a hemagglutinating *M. catarrhalis* strain has cytotoxic activity against a different hemagglutinating *M. catarrhalis* strain but not against a non-hemagglutinating *M. catarrhalis* strain.

3.1. DEFINITIONS AND ABBREVIATIONS

anti-OMP106	=	anti-OMP106 polypeptide antibody or antiserum
ATCC	=	American Type Culture Collection
blebs	=	naturally occurring outer membrane vesicles of *M. catarrhalis*
Gb_4	=	GalNAcβ1-3Galα1-4Galβ1-4Glc1-1Ceramide
HA	=	hemagglutinating
immuno-reactive	=	capable of provoking a cellular or humoral immune response
kD	=	kilodaltons

M. catarrhalis	=	*Mc*; *Moraxella catarrhalis*; *Moraxella (Branhamella) catarrhalis*; *Branhamella catarrhalis*; *Neisseria catarrhalis*; or *Micrococcus catarrhalis*
NHA	=	non-hemagglutinating
OG	=	n-octyl β-D-glucopyranoside or octyl glucoside
OMP106	=	the outer membrane protein-106 polypeptide of *Moraxella catarrhalis*, having a molecular weight of about 180 kD to 230 kD by SDS-PAGE; extractable from blebs or intact cells of *M. catarrhalis* by OG or sarkosyl detergent
OMP106-derived polypeptide	=	fragment of the OMP106 polypeptide; variant of wild-type OMP106 polypeptide or fragment thereof, containing one or more amino acid deletions, insertions or substitutions; or chimeric protein comprising a heterologous polypeptide fused to the C-terminal or N-terminal or internal segment of a whole or a portion of the OMP106 polypeptide
OMP	=	outer membrane protein
OMPs	=	outer membrane proteins
PBS	=	phosphate buffered saline
PAG	=	polyacrylamide gel
polypeptide	=	a peptide of any length, preferably one having ten or more amino acid residues
SDS	=	sodium dodecylsulfate

SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis

Nucleotide or nucleic acid sequences defined herein are represented by one-letter symbols for the bases as follows:

A (adenine)
C (cytosine)
G (guanine)
T (thymine)
U (uracil)
M (A or C)
R (A or G)
W (A or T/U)
S (C or G)
Y (C or T/U)
K (G or T/U)
V (A or C or G; not T/U)
H (A or C or T/U; not G)
D (A or G or T/U; not C)
B (C or G or T/U; not A)
N (A or C or G or T/U) or (unknown)

Peptide and polypeptide sequences defined herein are represented by one-letter symbols for amino acid residues as follows:

A (alanine)
R (arginine)
N (asparagine)
D (aspartic acid)
C (cysteine)
Q (glutamine)
E (glutamic acid)
G (glycine)
H (histidine)
I (isoleucine)

L (leucine)
K (lysine)
M (methionine)
F (phenylalanine)
P (proline)
S (serine)
T (threonine)
W (tryptophan)
Y (tyrosine)
V (valine)
X (unknown)

The present invention may be more fully understood by reference to the following detailed description of the invention, non-limiting examples of specific embodiments of the invention and the appended figures.

4. BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Denaturing PAGE comparison of outer membrane protein profiles of *M. catarrhalis* blebs or octyl glucoside (OG) extracts of whole *M. catarrhalis* cells. The numbers over the lanes refer to the ATCC strain designations. A prestained SDS-PAGE standard (BioRad catalog # 161-0305) was used as molecular weight markers. The standard consisted of the following polypeptides with their approximate molecular weights noted in parenthesis: rabbit muscle phosphorylase B (106 kD); bovine serum albumin (80 kD); hen egg white ovalbumin (49.5 kD); bovine carbonic anhydrase (32.5 kD); soybean trypsin inhibitor (27.5 kD); hen egg white lysozyme (18.5 kD). The positions of the molecular weight markers in the gel are noted on the left side of the drawing by arrows with the molecular weights (kD) of some of the markers above the arrows.

Fig. 2: Results from overlaying thin layer chromatograms of glycolipids with ^{125}I-labeled outer membrane blebs. In

Panels A-C, Lane 1 contains glycolipid standards indicated on the left; Lane 2 contains asialo-GM_1; Lane 3 contains Gb_3, Gb_4, and Forssman antigen; and Lane 4 contains a Folch extraction of human erythrocytes. The chromatogram shown in Panel A is stained with orcinol, the chromatogram shown in Panel B is overlayed with ^{125}I-labeled blebs of ATCC strain 8176 (a non-hemagglutinating strain), and the chromatogram shown in Panel C is overlayed with ^{125}I-labeled blebs of ATCC strain 49143 (a hemagglutinating strain). Only the hemagglutinating strain bound to the Gb_4 glycolipid band in the third and fourth lanes.

Fig. 3: Protein profiles by silver staining of octyl glucoside extracts of outer membrane proteins following digestion of the *M. catarrhalis* cells with the proteases indicated in the figure. The hemagglutination activity of the cells following the digestion is indicated below the figure in the row labeled HA. The molecular weight markers used are as per Fig. 1.

Fig. 4: Comparison of protein profiles by silver staining of outer membrane proteins from various ATCC strains of *M. catarrhalis*. The strain designations are indicated above the lanes. The hemagglutination activity of the strains are indicated in the row labeled HA below the figure. Note a protein having an apparent molecular weight greater than that of rabbit muscle phosphorylase B (106 kD) is common to the hemagglutinating strains, but is absent in the non-hemagglutinating strains. This polypeptide is designated OMP106. The molecular weight markers used are as per Fig. 1.

Fig. 5: Comparison of protein profiles by silver staining of outer membrane proteins from two *M. catarrhalis* ATCC 49143 cultivars: 49143 (hemagglutinating cultivar) and 49143-NHA (non-hemagglutinating cultivar). The hemagglutination activities of the cultivars are indicated below the figure in the row labeled HA. Note the absence of the OMP106

polypeptide band (indicated by <) in the non-hemagglutinating cultivar. The molecular weight markers used are as per Fig. 1.

Fig. 6: Molecular weight estimation of OMP106 in a 6% denaturing polyacrylamide gel using OG extracts of ATCC strain 49143 that were incubated in sample buffer at either 25°C or 100°C prior to application to the gel. Proteins in the gel were visualized by reductive silver staining. Note that the OMP106 polypeptide band (indicated by the <) is seen only in the sample incubated at 100°C. A broad range SDS-PAGE standard (BioRad catalog # 161-0317) was used as molecular weight markers. The standard consisted of the following polypeptides (approximate molecular weights noted in parenthesis): rabbit skeletal muscle myosin (200 kD); *E. coli* β-galactosidase (116 kD); rabbit muscle phosphorylase B (97.4 kD); bovine serum albumin (66.2 kD). The positions of the molecular weight markers in the gel are noted on the right side of the figure by arrows with the molecular weights (kD) of the markers above the arrows.

Fig. 7: Southern blot analysis of DraI and HindIII restriction endonuclease digests of *M. catarrhalis* chromosomal DNA probed with Mc5-72. DNA of *M. catarrhalis* strain 49143 was digested with DraI or HindIII. Southern analysis of the digested DNA was carried out using Mc5-72 (SEQ ID NO:4) as the probe. The high stringency wash was 2X SSC, 1% SDS at 50°C for about 20 to about 30 minutes. Lane 1 contains HindIII digest; the hybridizing band has an approximate size of 8.0 kB. Lane 2 contains DraI digest: the hybridizing band has an approximate size of 4.2 kB.

Figs. 8A and 8B: Western Blots of protein extracts of *M. catarrhalis* and related species using a rabbit antiserum to OMP106 as the probe (Fig. 8A), compared to the reactivity of the serum prior to immunization of the rabbit with OMP106 (Fig. 8B). Samples in the lanes of Figs. 8A and 8B are as

follows: Lane A, *M. catarrhalis*; Lane B, *Moraxella ovis*; Lane C, *Moraxella lacunata*; Lane D, *Moraxella osloensis*; Lane E, *Moraxella bovis*; Lane F, *Neisseria meningitidis*; Lane G, *Neisseria gonorrhoeae*. The molecular weight markers used are as per Fig. 1.

Fig. 9A. Western blot demonstrating that a rabbit antiserum to the OMP106 polypeptide from *M. catarrhalis* ATCC 49143 cross-reacts with a polypeptide of a similar molecular weight in a number of HA and NHA strains of *M. catarrhalis* (the location of the OMP106 polypeptide is indicated by the arrow). The Western examined octyl glucoside extracts of various *M. catarrhalis* strains. The ATCC accession numbers of the strains are indicated at the top of the lanes. The transfer and Western blot procedures used were identical to those used to obtain the blots shown in Fig. 8.

Fig. 9B. Western blot of the same extracts as those in Fig. 9A using the pre-immune serum corresponding to that used in Fig. 9A.

5. <u>DETAILED DESCRIPTION OF THE INVENTION</u>

5.1. <u>HEMAGGLUTINATING AND NON-HEMAGGLUTINATING CULTIVARS</u>

The invention provides an isolated or a substantially pure OMP106 polypeptide of *M. catarrhalis*. The OMP106 polypeptide comprises the whole or a subunit of a protein embedded in or located on the outer surface of the outer membrane of hemagglutinating (HA) strains and many nonhemagglutinating (NHA) strains and cultivars of *M. catarrhalis*. OMP106 contributes directly or indirectly to the hemagglutination phenotype of the HA strains and cultivars. According to the invention, HA *M. catarrhalis* cells agglutinate human or rabbit erythrocytes in any standard hemagglutination assay, such as the one taught by Soto-Hernandez et al. 1989, J. Clin. Microbiol. 27:903-908. Although not intending to be limited to any particular mechanism of action, it is presently envisaged that *M.*

catarrhalis agglutinates erythrocytes by binding to the globotetrose (Gb_4) moiety of glycolipid and glycoprotein receptors on the host cell surfaces and that the hemagglutination activity is mediated in part by appropriately modified OMP106 polypeptide, which has the particular property of being susceptible to silver staining. By contrast, unmodified or inappropriately modified OMP106 polypeptide is neither active in mediating hemagglutination nor silver-stainable. Moreover, OMP106 polypeptide is the only polypeptide having an apparent molecular weight of about 180 kD to about 230 kD in SDS-PAGE that is OG- or sarkosyl-extractable from HA or NHA *M. catarrhalis* blebs or intact cells.

The hemagglutination activity of HA *M. catarrhalis* cells is inhibited by globotetrose (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1; Gb_4) and the monosaccharides that comprise Gb4, including N-acetyl-D-galactosamine, D-galactose and glucose, and derivatives thereof, such as methyl-α-galactose or methyl-ß-galactose. The hemagglutination activity of HA *M. catarrhalis* cells is also inhibited by relatively higher concentrations of a number of other sugars including but not limited to D-mannose, L-fucose, D-glucose, and N-acetyl-D-glucosamine.

The hemagglutination activity and the OMP106 polypeptide of intact HA *M. catarrhalis* cells are both reduced or destroyed by digestion of intact *M. catarrhalis* cells by various proteases including, but not limited to, TLCK (Nα-ptosyl-L-lysine chloro methyl ketone [also known as 1-chloro-3-tosylamino-7-amino-L-2-heptanone])-treated chymotrypsin, proteinase K and TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin. Protease V8 digestion of intact HA *M. catarrhalis* cells, however, affects neither the hemagglutination activity nor the physical integrity of the OMP106 polypeptide of such cells.

A non-hemagglutinating (NHA) cultivar may be derived from a HA *M. catarrhalis* strain or cultivar by serial passage in static liquid cultures (i.e., liquid cultures

maintained at 35°C without shaking). For example, a HA *M. catarrhalis* strain or cultivar is grown in Mueller Hinton broth and every five days an inoculum is taken from the surface of the static culture to inoculate a subsequent static culture. The preferred inoculum is any floating mat of cells at the surface of the culture. Passaging in static cultures is maintained until a NHA cultivar is produced. A NHA cultivar of the invention may be used to produce protective vaccines, such as whole cell vaccines, against *M. catarrhalis* infections.

By contrast, the hemagglutinating phenotype of a HA *M. catarrhalis* strain or cultivar can be maintained by passaging the strain or cultivar in shaking liquid cultures. In an embodiment, a HA *M. catarrhalis* strain or cultivar is grown in Mueller Hinton broth at 35 to 37°C with shaking at about 200 RPM and passaged every 24 to 48 hours. The hemagglutinating phenotype of a HA *M. catarrhalis* strain or cultivar also can be maintained by passaging on solid media. For example, a HA *M. catarrhalis* strain or cultivar is grown on a plate containing blood agar or Mueller Hinton agar.

5.2. OMP106 POLYPEPTIDE

OMP106 polypeptide of the invention is the sole outer membrane protein of a HA *M. catarrhalis* strain or cultivar that has an apparent molecular weight in SDS-PAGE of about 180 kD to about 230 kD, preferably about 190 kD. According to the invention, an outer membrane protein of *M. catarrhalis* is a polypeptide that is present in *M. catarrhalis* blebs, or that can be extracted from *M. catarrhalis* blebs or intact cells by n-octyl β-D-glucopyranoside (OG) or sarkosyl detergent in buffer solution at room temperature. See Murphy and Loeb, 1989, Microbial Pathogenesis 6:159-174, for a discussion of *M. catarrhalis* blebs, which are naturally occurring vesicles consisting of the outer membrane of *M. catarrhalis*. NHA *M. catarrhalis* strains or cultivars either do not have OMP106 polypeptide, or have OMP106 polypeptide in a form that binds anti-OMP106

antibodies (see Section 5.5., *infra*) but does not react with silver stain (i.e., using Silver Stain Plus of BioRad [Richmond, CA], or the procedure described by Gottlieb and Chauko, 1987, Anal. Biochem. 165:33). By contrast, OMP106 polypeptide from HA *M. catarrhalis* strains or cultivars binds anti-OMP106 antibodies, and reacts with silver stain.

OMP106 polypeptide may be identified in HA *M. catarrhalis* blebs or intact cells by its susceptibility to degradation by protease treatment that also abolishes or attenuates the hemagglutination activity of the same HA strain (See Section 5.1. above for examples of proteases that do or do not destroy hemagglutination activity of intact *M. catarrhalis* cells). In other words, digestion with a protease that destroys or reduces the hemagglutination activity of a HA strain or cultivar will also change, in SDS-PAGE, the abundance or the location of OMP106 polypeptide isolated from the strain or cultivar after such a digestion as compared to that isolated from the same strain or cultivar before the digestion.

OMP106 polypeptide may also be identified as the polypeptide in OG or sarkosyl extract of *M. catarrhalis* blebs or intact cells that has an apparent molecular weight of greater than 106 kD as determined by denaturing gel electrophoresis in 12% PAG with SDS, using formulations as described in Harlow and Lane (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix I, 1988). Heat treatment of the OG or sarkosyl extract at 100°C for 5 minutes can cause the OMP106 polypeptide to have an apparent molecular weight of about 180 kD to about 230 kD as determined by SDS-PAGE in 6% PAG without any reducing agents, using formulations as described in Harlow and Lane, *id*. In a particular embodiment, OMP106 polypeptide in the heat-treated OG or sarkosyl extract of *M. catarrhalis* strain ATCC 49143 has an apparent molecular weight of about 190 kD.

In particular embodiments, the OMP106 polypeptide is that prepared from any of *M. catarrhalis* strains

including, but not limited to, ATCC 49143, ATCC 25238, ATCC 25240, ATCC 43617, ATCC 43618, ATCC 43627 and ATCC 43628. The preferred source of OMP106 polypeptide is a HA cultivar of such strains. The more preferred source is a HA cultivar of ATCC 49143.

In a particular embodiment, OMP106 polypeptide comprises, preferably at the amino-terminal, the amino acid sequence IGISEADGGKGGANARGDKSIAIGDIAQALGSQSIAIGDNKIV (SEQ ID NO:1) or a sequence substantially homologous thereto. The OMP106 polypeptide may additionally comprise, carboxyl-distal to the above mentioned sequence, an octapeptide having the amino acid sequence GTVLGGKK (SEQ ID NO:2) or a sequence substantially homologous thereto. As used herein a substantially homologous amino acid sequence is at least 80%, preferably 100%, identical to the referenced amino acid sequence.

According to various aspects of the invention, the polypeptides of the invention are characterized by their apparent molecular weights based on the polypeptides' migration in SDS-PAGE relative to the migration of known molecular weight markers. While any molecular weight standards known in the art may be used with the SDS-PAGE, preferred molecular weight markers comprise at least rabbit skeletal muscle myosin, *E. coli* β-galactosidase and rabbit muscle phosphorylase B. One skilled in the art will appreciate that the polypeptides of the invention may migrate differently in different types of gel systems (e.g., different buffers; different concentration of gel, crosslinker or SDS). One skilled in the art will also appreciate that the polypeptides may have different apparent molecular weights due to different molecular weight markers used with the SDS-PAGE. Hence, the molecular weight characterization of the polypeptides of the invention is intended to be directed to cover the same polypeptides on any SDS-PAGE systems and with any molecular weight markers which might indicate sightly different apparent molecular weights for the polypeptides than those disclosed here.

5.3. OMP106-DERIVED POLYPEPTIDES

An OMP106-derived polypeptide of the invention may be a fragment of the OMP106 polypeptide. The intact OMP106 polypeptide may contain one or more amino acid residues that are not necessary to its immunogenicity. It may be the case, for example, that only the amino acid residues forming a particular epitope of the OMP106 polypeptide is necessary for immunogenic activity. Unnecessary amino acid sequences can be removed by techniques well-known in the art. For example, the unwanted amino acid sequences can be removed by limited proteolytic digestion using enzymes such as trypsin, papain, or related proteolytic enzymes or by chemical cleavage using agents such as cyanogen bromide and followed by fractionation of the digestion or cleavage products.

An OMP106-derived polypeptide of the invention may also be a modified OMP106 polypeptide or fragment thereof (i.e., an OMP106 polypeptide or fragment having one or more amino acid substitutions, insertions and/or deletions of the wild-type OMP106 sequence). Such modifications may enhance the immunogenicity of the resultant polypeptide product or have no effect on such activity. Modification techniques that may be used include those disclosed in U.S. Patent No. 4,526,716.

An OMP106-derived polypeptide may further be a chimeric polypeptide comprising one or more heterologous polypeptides fused to the amino-terminal or carboxyl-terminal or internal of a complete OMP106 polypeptide or a portion of or a fragment thereof. Useful heterologous polypeptides comprising such chimeric polypeptide include, but are not limited to, a) pre- and/or pro- sequences that facilitate the transport, translocation and/or processing of the OMP106-derived polypeptide in a host cell, b) affinity purification sequences, and c) any useful immunogenic sequences (e.g., sequences encoding one or more epitopes of a surface-exposed protein of a microbial pathogen).

Preferably, the OMP106-derived polypeptides of the invention are immunologically cross-reactive with the OMP106

polypeptide, thus being capable of eliciting in an animal an immune response to *M. catarrhalis*. More preferably, the OMP106-derived polypeptides of the invention comprise sequences forming one or more outer-surface epitopes of the native OMP106 polypeptide of *M. catarrhalis* (i.e., the surface-exposed epitopes of OMP106 polypeptide as it exists in intact *M. catarrhalis* cells). Such preferred OMP106-derived polypeptides can be identified by their ability to specifically bind antibodies raised to intact *M. catarrhalis* cells (e.g., antibodies elicited by formaldehyde or glutaldehyde fixed *M. catarrhalis* cells; such antibodies are referred to herein as "anti-whole cell" antibodies). For example, polypeptides or peptides from a limited or complete protease digestion of the OMP106 polypeptide are fractionated using standard methods and tested for their ability to bind anti-whole cell antibodies. Reactive polypeptides comprise preferred OMP106-derived polypeptides. They are isolated and their amino acid sequences determined by methods known in the art.

Also preferably, the OMP106-derived polypeptides of the invention comprise sequences that form one or more epitopes of native OMP106 polypeptide that mediate hemagglutination by HA *M. catarrhalis* cells. Such preferred OMP106-derived polypeptides may be identified by their ability to interfere with hemagglutination by HA *M. catarrhalis* cells. For example, polypeptides from a limited or complete protease digestion or chemical cleavage of OMP106 polypeptide are fractionated using standard methods and tested for the ability to interfere in hemagglutination by *M. catarrhalis* cells. Once identified and isolated the amino acid sequences of such preferred OMP106-derived polypeptides are determined using standard sequencing methods. The determined sequence may be used to enable production of such polypeptides by synthetic chemical and/or genetic-engineering means.

These preferred OMP106-derived polypeptides also can be identified by using anti-whole cell antibodies to

screen bacterial libraries expressing random fragments of *M. catarrhalis* genomic DNA or cloned nucleotide sequences encoding the OMP106 polypeptide. See, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, NY, Vol. 1, Chapter 12. The reactive clones are identified and their inserts are isolated and sequenced to determine the amino acid sequences of such preferred OMP106-derived polypeptides.

5.4. ISOLATION AND PURIFICATION OF OMP106 POLYPEPTIDE AND OMP106-DERIVED POLYPEPTIDES

The invention provides isolated OMP106 polypeptides and OMP106-derived polypeptides. As used herein, the term "isolated" means that the product is significantly free of other biological materials with which it is naturally associated. That is, for example, an isolated OMP106 polypeptide composition is between about 70% and 94% pure OMP106 polypeptide by weight. Preferably, the OMP106 polypeptides and OMP106-derived polypeptides of the invention are purified. As used herein, the term "purified" means that the product is substantially free of other biological material with which it is naturally associated. That is comprising a purified OMP106 polypeptide composition is at least 95% pure OMP106 polypeptide by weight, preferably at least 98% pure OMP106 polypeptide by weight, and most preferably at least 99% pure OMP106 polypeptide by weight.

The OMP106 polypeptide of the invention may be isolated from protein extracts including whole cell extract, of any *M. catarrhalis* strain or cultivar. Preferably, the protein extract is an octyl glucoside or sarkosyl extract of outer membrane vesicles (i.e., blebs) or whole cells of *M. catarrhalis* including, but not limited to, any of strains ATCC 49143, ATCC 25238, ATCC 25240, ATCC 43617, ATCC 43618, ATCC 43627 and ATCC 43628. The preferred source of such extracts is a HA cultivar of such strains. The more preferred source of such extracts is a HA cultivar of ATCC 49143. Another source of the OMP106 polypeptide is protein

preparations from gene expression systems expressing cloned sequences encoding OMP106 polypeptide or OMP106-derived polypeptides (see Section 5.8., *infra*).

The OMP106 polypeptide can be isolated and purified from the source material using any biochemical technique and approach well known to those skilled in the art. In one approach, *M. catarrhalis* outer membrane is obtained by standard techniques and outer membrane proteins are solubilized using a solubilizing compound such as a detergent. A preferred solubilizing solution is one containing about 1.25% octyl glucopyranoside w/v (OG). Another preferred solubilizing solution is one containing about 1.25% sarkosyl. OMP106 polypeptide is in the solubilized fraction. Cellular debris and insoluble material in the extract are separated and removed preferably by centrifuging. The polypeptides in the extract are concentrated, incubated in SDS-containing Laemmli gel sample buffer at 100°C for 5 minutes and then fractionated by electrophoresis in a 6% denaturing sodium dodecylsulfate (SDS) polyacrylamide gel (PAG) without reducing agent. See Laemmli, 1970, Nature 227:680-685. The band or fraction identified as OMP106 polypeptide as described above (e.g., the silver-stained polypeptide band that is present in the OG or sarkosyl extract of a HA but not that of a corresponding NHA cultivar or that of the HA cultivar after digestion with a protease that abolishes hemagglutination activity) may then be isolated directly from the fraction or gel slice containing the OMP106 polypeptide. In a preferred embodiment, OMP106 polypeptide has an apparent molecular weight of 190 kD as determined by comparing its migration distance or rate in a denaturing SDS-PAGE relative to those of rabbit skeletal muscle myosin (200 kD) and *E. coli* β-galactosidase (116 kD).

Another method of purifying OMP106 polypeptide is by affinity chromatography using anti-OMP106 antibodies, (see Section 5.5). Preferably, monoclonal anti-OMP106 antibodies are used. The antibodies are covalently linked to agarose

gels activated by cyanogen bromide or succinamide esters (Affi-Gel, BioRad, Inc.) or by other methods known to those skilled in the art. The protein extract is loaded on the top of the gel as described above. The contact is for a period of time and under standard reaction conditions sufficient for OMP106 polypeptide to bind to the antibody. Preferably, the solid support is a material used in a chromatographic column. OMP106 polypeptide is then removed from the antibody, thereby permitting the recovery OMP106 polypeptide in isolated, or preferably, purified form.

An OMP106-derived polypeptide of the invention can be produced by chemical and/or enzymatic cleavage or degradation of isolated or purified OMP106 polypeptide. An OMP106-derived polypeptide can also be chemically synthesized based on the known amino acid sequence of OMP106 polypeptide and, in the case of a chimeric polypeptide, those of the heterologous polypeptide by methods well-known in the art. See, for example, Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman and Co., NY.

An OMP106-derived polypeptide can also be produced in a gene expression system expressing a recombinant nucleotide construct comprising sequences encoding OMP106-derived polypeptides. The nucleotide sequences encoding polypeptides of the invention may be synthesized, and/or cloned, and expressed according to techniques well known to those skilled in the art. See, for example, Sambrook, et al., 1989, Molecular Cloning, A Laboratory Manual, Vols. 1-3, Cold Spring Harbor Press, NY, Chapter 9.

OMP106-derived polypeptides of the invention can be fractionated and purified by the application of standard protein purification techniques, modified and applied in accordance with the discoveries and teachings described herein. In particular, preferred OMP106-polypeptides of the invention, those that form an outer-surface epitope of the native OMP106 polypeptide may be isolated and purified according to the affinity procedures disclosed above for the

isolation and purification of OMP106 polypeptide (e.g., affinity purification using anti-OMP106 antibodies.

If desirable, the polypeptides of the invention may be further purified using standard protein or peptide purification techniques including but are not limited to electrophoresis, centrifugation, gel filtration, precipitation, dialysis, chromatography (including ion exchange chromatography, affinity chromatography, immunoadsorbent affinity chromatography, reverse-phase high performance liquid chromatography, and gel permeation high performance liquid chromatography), isoelectric focusing, and variations and combinations thereof.

One or more of these techniques may be employed sequentially in a procedure designed to separate molecules according to their physical or chemical characteristics. These characteristics include the hydrophobicity, charge, binding capability, and molecular weight of the protein. The various fractions of materials obtained after each technique are tested for their abilities to bind the OMP106 receptor or ligand, to bind anti-OMP106 antibodies or to interfere with hemagglutination by HA *M. catarrhalis* cells ("test" activities). Those fractions showing such activity are then subjected to the next technique in the sequential procedure, and the new fractions are tested again. The process is repeated until only one fraction having the above described "test" activities remains and that fraction produces only a single band or entity when subjected to polyacrylamide gel electrophoresis or chromatography.

5.5. OMP106 IMMUNOGENS AND ANTI-OMP106 ANTIBODIES

The present invention provides antibodies that specifically bind OMP106 polypeptide or OMP106-derived polypeptides. For the production of such antibodies, isolated or preferably, purified preparations of OMP106 polypeptide or OMP106-derived polypeptides are used as immunogens.

In an embodiment, the OMP106 polypeptide is separated from other outer membrane proteins present in the OG or sarksyl extract of outer membrane of HA *M. catarrhalis* cells or blebs using SDS-PAGE (see Section 5.2. above) and the gel slice containing OMP106 polypeptide is used as the immunogen and injected into a rabbit to produce antisera containing polyclonal OMP106 antibodies. The same immunogen can be used to immunize mice for the production of hybridoma lines that produce monoclonal anti-OMP106 antibodies. In particular embodiments, a PAG slice containing isolated or purified OMP106 from any of strains ATCC 49143, ATCC 25238, ATCC 25240, ATCC 43617, ATCC 43618, ATCC 43627 and ATCC 43628 is used as the immunogen. In preferred embodiments, a PAG slice containing isolated or purified OMP106 from a HA cultivar of such strains is used. In a more preferred embodiment, a PAG slice containing isolated or purified OMP106 from a HA cultivar of strain ATCC 49143 is used as the immunogen.

In other embodiments, peptide fragments of OMP106 polypeptide are used as immunogens. Preferably, peptide fragments of purified OMP106 polypeptide are used. The peptides may be produced by protease digestion, chemical cleavage of isolated or purified OMP106 polypeptide or chemical synthesis and then may be isolated or purified. Such isolated or purified peptides can be used directly as immunogens. In particular embodiments, useful peptide fragments include but are not limited to those having the sequence IGISEADGGKGGANARGDKSIAIGDIAQALGSQSIAIGDNKIV (SEQ ID NO:1) or any portion thereof that is 6 or more amino acids in length. In an another embodiment, the peptide fragment has the sequence GTVLGGKK (SEQ ID NO:2).

Useful immunogens may also comprise such peptides or peptide fragments conjugated to a carrier molecule, preferably a carrier protein. Carrier proteins may be any commonly used in immunology, include, but are not limited to, bovine serum albumin (BSA), chicken albumin, keyhole limpet hemocyanin (KLH) and the like. For a discussion of hapten

protein conjugates, see, for example, Hartlow, et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988, or a standard immunology textbook such as Roitt, I. et al., IMMUNOLOGY, C.V. Mosby Co., St. Louis, MO (1985) or Klein, J., IMMUNOLOGY, Blackwell Scientific Publications, Inc., Cambridge, MA, (1990).

In yet another embodiment, for the production of antibodies that specifically bind one or more outer-surface epitopes of the native OMP106 polypeptide, intact HA *M. catarrhalis* cells or blebs prepared therefrom are used as immunogen. The cells or blebs may be fixed with agents such as formaldehyde or glutaldehyde before immunization. See Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988, Chapter 15. It is preferred that such anti-whole cell antibodies be monoclonal antibodies. Hybridoma lines producing the desired monoclonal antibodies can be identified by using purified OMP106 polypeptide as the screening ligand. Cells or blebs of any *M. catarrhalis* strain including, but not limited to, ATCC 49143, ATCC 25238, ATCC 25240, ATCC 43617, ATCC 43618, ATCC 43627 and ATCC 43628 are used as the immunogen for inducing these antibodies. Preferably, cells or blebs of a HA cultivar of such strains are used as the immunogen. More preferably, cells or blebs of a HA cultivar of strain ATCC 49143 are used as the immunogen for inducing these antibodies.

Polyclonal antibodies produced by whole cell or bleb immunizations contain antibodies that bind other *M. catarrhalis* outer membrane proteins ("non-anti-OMP106 antibodies") and thus are more cumbersome to use where it is known or suspected that the sample contains other *M. catarrhalis* outer membrane proteins or materials that are cross-reactive with these other outer membrane proteins. Under such circumstances, any binding by the anti-whole cell antibodies of a given sample or band must be verified by coincidental binding of the same sample or band by antibodies

that specifically bind OMP106 polypeptide (e.g., anti-OMP106) and/or a OMP106-derived polypeptide, or by competition tests using anti-OMP106 antibodies, OMP106 polypeptide or OMP106-derived polypeptide as the competitor (i.e., addition of anti-OMP106 antibodies, OMP106 polypeptide or OMP106-derived polypeptide to the reaction mix lowers or abolishes sample binding by anti-whole cell antibodies). Alternatively, such polyclonal antisera, containing "non-anti-OMP106" antibodies, may be cleared of such antibodies by standard approaches and methods. For example, the non-anti-OMP106 antibodies may be removed by precipitation with cells of NHA *M. catarrhalis* cultivars or *M. catarrhalis* strains known not to have the OMP106 polypeptide (e.g., ATCC 8176, more preferably a NHA cultivar of ATCC 49143); or by absorption to columns comprising such cells or outer membrane proteins of such cells.

In further embodiments, useful immunogens for eliciting antibodies of the invention comprise mixtures of two or more of any of the above-mentioned individual immunogens.

Immunization of mammals with the immunogens described herein, preferably humans, rabbits, rats, mice, sheep, goats, cows or horses, is performed following procedures well known to those skilled in the art, for purposes of obtaining antisera containing polyclonal antibodies or hybridoma lines secreting monoclonal antibodies.

Monoclonal antibodies can be prepared by standard techniques, given the teachings contained herein. Such techniques are disclosed, for example, in U.S. Patent No. 4,271,145 and U.S. Patent No. 4,196,265. Briefly, an animal is immunized with the immunogen. Hybridomas are prepared by fusing spleen cells from the immunized animal with myeloma cells. The fusion products are screened for those producing antibodies that bind to the immunogen. The positive hybridomas clones are isolated, and the monoclonal antibodies are recovered from those clones.

Immunization regimens for production of both polyclonal and monoclonal antibodies are well-known in the art. The immunogen may be injected by any of a number of routes, including subcutaneous, intravenous, intraperitoneal, intradermal, intramuscular, mucosal, or a combination of these. The immunogen may be injected in soluble form, aggregate form, attached to a physical carrier, or mixed with an adjuvant, using methods and materials well-known in the art. The antisera and antibodies may be purified using column chromatography methods well known to those of skill in the art.

According to the present invention, OMP106 polypeptides of *M. catarrhalis* strains, HA or NHA, are immuno-cross reactive. Thus, antibodies raised to OMP106 polypeptide of one *M. catarrhalis* strain or cultivar specifically bind OMP106 polypeptide and OMP106-derived polypeptides of other *M. catarrhalis* strains and cultivars. For example, polyclonal anti-OMP106 antibodies induced by OMP106 polypeptide of strain ATCC 49143 specifically bind not only the homologous OMP106 polypeptide (i.e., the OMP106 polypeptide of strain ATCC 49143) but also OMP106 polypeptide and/or OMP106-derived polypeptides of other *M. catarrhalis* strains including, but not limited to, ATCC 43628, ATCC 43627, ATCC 43618, ATCC 43617, ATCC 25240 and ATCC 25238.

The antibodies of the invention, including but not limited to anti-OMP106 antibodies, can be used to facilitate isolation and purification of OMP106 polypeptide and OMP106-derived polypeptides. The antibodies may also be used as probes for identifying clones in expression libraries that have inserts encoding OMP106 polypeptide or fragments thereof. The antibodies may also be used in immunoassays (e.g., ELISA, RIA, Westerns) to specifically detect and/or quantitate *M. catarrhalis* in biological specimens. Anti-OMP106 antibodies of the invention specifically bind OMP106 polypeptide and do not bind proteins from related bacterial pathogens such as *Moraxella ovis*, *Moraxella lacunata*, *Moraxella osloensis*, *Moraxella bovis*, *Neisseria meningitidis*,

Neisseria gonorrhoeae. Thus anti-OMP106 antibodies can be used to diagnose *M. catarrhalis* infections.

The antibodies of the invention, particularly those which are cytotoxic, may also be used in passive immunization to prevent or attenuate *M. catarrhalis* infections of animals, including humans. (As used herein, a cytotoxic antibody is one which enhances opsinization and/or complement killing of the bacterium bound by the antibody) An effective concentration of polyclonal or monoclonal antibodies raised against the immunogens of the invention may be administered to a host to achieve such effects. The exact concentration of the antibodies administered will vary according to each specific antibody preparation, but may be determined using standard techniques well known to those of ordinary skill in the art. Administration of the antibodies may be accomplished using a variety of techniques, including, but not limited to those described in Section 5.6. for the delivery of vaccines.

Prophylactic and therapeutic efficacies of the antibodies of the invention can be determined by standard pharmaceutical procedures in experimental animals. The data obtained from animal studies can be used in formulating a range of dosages for use in humans.

5.6. VACCINES

The present invention also provides therapeutic and prophylactic vaccines against *M. catarrhalis* infections of animals, including mammals, and more specifically rodents, primates, and humans. The preferred use of the vaccines is in humans. The vaccines can be prepared by techniques known to those skilled in the art and would comprise, for example, the antigen in form of an immunogen, a pharmaceutically acceptable carrier, possibly an appropriate adjuvant, and possibly other materials traditionally found in vaccines. An immunologically effective amount of the immunogen to be used in the vaccine is determined by means known in the art in view of the teachings herein.

The vaccines of the present invention comprise an immunologically effective amount of any of the immunogens disclosed in Section 5.5. in a pharmaceutically acceptable carrier.

According to another embodiment, the vaccines of the invention comprise an immunologically effective amount of an inactivated or attenuated HA *M. catarrhalis* cultivar or NHA *M. catarrhalis* cultivar of the invention. An inactivated or attenuated HA *M. catarrhalis* cultivar or NHA *M. catarrhalis* cultivar is obtained using any methods known in the art including, but not limited to, chemical treatment (e.g., formalin), heat treatment and irradiation.

The term "immunologically effective amount" is used herein to mean an amount sufficient to induce an immune response which can prevent *M. catarrhalis* infections or attenuate the severity of any preexisting or subsequent *M. catarrhalis* infections. The exact concentration will depend upon the specific immunogen to be administered, but may be determined by using standard techniques well known to those skilled in the art for assaying the development of an immune response.

Useful polypeptide immunogens include the isolated OMP106 polypeptide and OMP106-derived polypeptides. Preferred immunogens include the purified OMP106 polypeptide and derived polypeptides or peptides of OMP106. The combined immunogen and carrier may be an aqueous solution, emulsion or suspension. In general, the quantity of polypeptide immunogen will be between 0.1 and 500 micrograms per dose. The carriers are known to those skilled in the art and include stabilizers, diluents, and buffers. Suitable stabilizers include carbohydrates, such as sorbitol, lactose, manitol, starch, sucrose, dextran, and glucose and proteins, such as albumin or casein. Suitable diluents include saline, Hanks Balanced Salts, and Ringers solution. Suitable buffers include an alkali metal phosphate, an alkali metal carbonate, or an alkaline earth metal carbonate. The vaccine may also contain one or more adjuvants to improve or enhance the

immunological response. Suitable adjuvants include, but are not limited to, peptides; aluminum hydroxide; aluminum phosphate; aluminum oxide; a composition that consists of a mineral oil, such as Marcol 52, or a vegetable oil and one or more emulsifying agents, or surface active substances such as lysolecithin, polycations, polyanions; and potentially useful human adjuvants such as BCG and *Corynebacterium parvum*. The vaccine may also contain other immunogens. Such a cocktail vaccine has the advantage that immunity against several pathogens can be obtained by a single administration. Examples of other immunogens are those used in the known DPT vaccines.

The vaccines of the invention are prepared by techniques known to those skilled in the art, given the teachings contained herein. Generally, an immunogen is mixed with the carrier to form a solution, suspension, or emulsion. One or more of the additives discussed above may be in the carrier or may be added subsequently. The vaccine preparations may be desiccated, for example, by freeze drying for storage purposes. If so, they may be subsequently reconstituted into liquid vaccines by the addition of an appropriate liquid carrier.

The vaccines are administered to humans or other mammals, including rodents and primates. They can be administered in one or more doses. The vaccines may be administered by known routes of administration. Many methods may be used to introduce the vaccine formulations described here. These methods include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. The preferred routes are intramuscular or subcutaneous injection.

The invention also provides a method for inducing an immune response to *M. catarrhalis* in a mammal in order to protect the mammal against infection and/or attenuate disease caused by *M. catarrhalis*. The method comprises administering an immunologically effective amount of the immunogens of the

invention to the host and, preferably, administering the vaccines of the invention to the host.

5.7. NUCLEIC ACIDS ENCODING OMP106 POLYPEPTIDE AND OMP106-DERIVED POLYPEPTIDES

The present invention also provides nucleic acids, DNA and RNA, encoding OMP106 polypeptide and OMP106-derived polypeptides. In one aspect, the nucleic acids of the invention may be synthesized using methods known in the art. Specifically, a portion of or the entire amino acid sequence of OMP106 polypeptide or an OMP106-derived polypeptide may be determined using techniques well known to those of skill in the art, such as via the Edman degradation technique (see, e.g., Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence obtained is used as a guide for the synthesis of DNA encoding OMP106 polypeptide or OMP106-derived polypeptide using conventional chemical approaches or polymerase chain reaction (PCR) amplification of overlapping oligonucleotides.

In another aspect, the amino acid sequence may be used as a guide for synthesis of oligonucleotide mixtures which in turn can be used to screen for OMP106 polypeptide coding sequences in *M. catarrhalis* genomic libraries. Such libraries may be prepared by isolating DNA from cells of any *M. catarrhalis* strain. Preferably the DNA used as the source of the OMP106 polypeptide coding sequence, for both genomic libraries and PCR amplification, is prepared from cells of any *M. catarrhalis* strain including, but not limited to, ATCC 49143, ATCC 25238, ATCC 25240, ATCC 43617, ATCC 43618, ATCC 43627 and ATCC 43628.

In the preparation of genomic libraries, DNA fragments are generated, some of which will encode parts or the whole of *M. catarrhalis* OMP106 polypeptide. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically

sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation. The DNA fragments can then be inserted into suitable vectors, including but not limited to plasmids, cosmids, bacteriophages lambda or T_4, and yeast artificial chromosome (YAC). (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) The genomic library may be screened by nucleic acid hybridization to labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961).

The genomic libraries may be screened with a labeled degenerate oligonucleotide corresponding to the amino acid sequence of any peptide of OMP106 polypeptide using optimal approaches well known in the art. In particular embodiments, the screening probe is a degenerate oligonucleotide that corresponds to the peptide having the sequence IGISEADGGKGGANARGDKSIAIGDIAQALGSQSIAIGDNKIV (SEQ ID NO:1) or a portion thereof. In another embodiment the screening probe may be a degenerate oligonucleotide that corresponds to a peptide having the sequence GTVLGGKK (SEQ ID NO:2). In an additional embodiment, the oligonucleotides GGNACNGTNCTNGGNGGNAARAAR (SEQ ID NO:3) and GGNACNGTNTTRGGNGGNAARAAR (SEQ ID NO:7), each corresponding to OMP106 peptide GTVLGGKK (SEQ ID NO:2), is used as the probe. In further embodiments, the sequence GAAGCGGACGGGGGGAAAGGCGGAGCCAATGCGCGCGGTGATAAATCCATTGCTATTGGTGACATTGCGCAA (SEQ ID NO:4) or any fragments thereof, or any complement of the sequence or fragments may be used as the probe. Any probe used preferably is 15 nucleotides or longer.

Clones in libraries with insert DNA encoding the OMP106 polypeptide or fragments thereof will hybridize to one or more of the degenerate oligonucleotide probes. Hybridization of such oligonucleotide probes to genomic libraries are carried out using methods known in the art. For example, hybridization with the two above-mentioned oligonucleotide probes may be carried out in 2X SSC, 1.0% SDS at 50°C and washed using the same conditions. In a particular embodiment, ATCC 49143 DNA sequence encoding the whole or a part of the OMP106 polypeptide is a HindIII restriction fragment of about 8,000 bp in length or a DRAI restriction fragment of about 4,200 bp in length.

In yet another aspect, clones of nucleotide sequences encoding a part or the entire OMP106 polypeptide or OMP106-derived polypeptides may also be obtained by screening *M. catarrhalis* expression libraries. For example, *M. catarrhalis* DNA is isolated and random fragments are prepared and ligated into an expression vector (*e.g.*, a bacteriophage, plasmid, phagemid or cosmid) such that the inserted sequence in the vector is capable of being expressed by the host cell into which the vector is then introduced. Various screening assays can then be used to select for the expressed OMP106 polypeptide or OMP106-derived polypeptides. In one embodiment, the various anti-OMP106 antibodies of the invention (see Section 5.5) can be used to identify the desired clones using methods known in the art. See, for example, Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix IV. Clones or plaques from the library are brought into contact with the antibodies to identify those clones that bind.

In an embodiment, colonies or plaques containing DNA that encodes OMP106 polypeptide or OMP106-derived polypeptide could be detected using DYNA Beads according to Olsvick et al., 29th ICAAC, Houston, Tex. 1989, incorporated herein by reference. Anti-OMP106 antibodies are crosslinked to tosylated DYNA Beads M280, and these antibody-containing

beads would then be used to adsorb to colonies or plaques expressing OMP106 polypeptide or OMP106-derived polypeptide. Colonies or plaques expressing OMP106 polypeptide or OMP106-derived polypeptide is identified as any of those that bind the beads.

Alternatively, the anti-OMP106 antibodies can be nonspecifically immobilized to a suitable support, such as silica or Celite™ resin. This material would then be used to adsorb to bacterial colonies expressing OMP106 polypeptide or OMP106-derived polypeptide as described in the preceding paragraph.

In another aspect, PCR amplification may be used to produce substantially pure DNA encoding a part of or the whole of OMP106 polypeptide from *M. catarrhalis* genomic DNA. Oligonucleotide primers, degenerate or otherwise, corresponding to known OMP106 polypeptide sequences can be used as primers. In particular embodiments, an oligonucleotide, degenerate or otherwise, encoding the peptide IGISEADGGKGGANARGDKSIAIGDIAQALGSQSIAIGDNKIV (SEQ ID NO:1) or any portion thereof may be used as the 5′ primer. For example, a 5′ primer may be the nucleotide sequence GAAGCGGACGGGGGAAAGGCGGAGCCAATGCGCGCGGTGATAAATCCATTGCTATTGGTGACATTGCGCAA (SEQ ID NO:4) or any portion thereof. Nucleotide sequences, degenerate or otherwise, that are reverse complements of sequence encoding GTVLGGKK (SEQ ID NO:2) may be used as the 3′ primer. For example, an oligonucleotide, degenerate or otherwise, that has the degenerate nucleotide sequence YTTYTTNCCNCCNAGNACNGTNCC (SEQ ID NO:6) or YTTYTTNCCNCCYAANACNGTNCC (SEQ ID NO:8) may be used as the 3′ primer in conjunction with the various 5′ primer discussed above.

PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser

degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in *M. catarrhalis* DNA. After successful amplification of a segment of the sequence encoding OMP106 polypeptide, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*.

Once an OMP106 polypeptide coding sequence has been isolated from one *M. catarrhalis* strain or cultivar, it is possible to use the same approach to isolate OMP106 polypeptide coding sequences from other *M. catarrhalis* strains and cultivars. It will be recognized by those skilled in the art that the DNA or RNA sequence encoding OMP106 polypeptide (or fragments thereof) of the invention can be used to obtain other DNA or RNA sequences that hybridize with it under conditions of moderate to high stringency, using general techniques known in the art. Hybridization with an OMP106 sequence from one *M. catarrhalis* strain or cultivar under high stringency conditions will identify the corresponding sequence from other strains and cultivars. High stringency conditions vary with probe length and base composition. The formula for determining such conditions are well known in the art. See Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, NY, Chapter 11. As used herein high stringency hybridization conditions as applied to probes of greater than 300 bases in length involve a final wash in 0.1X SSC/0.1% SDS at 68°C for at least 1 hour (Ausubel, et al., Eds., 1989, Current Protocols in Molecular Biology, Vol. I, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., New York, at page 2.10.3). In particular embodiments, the high stringency wash in hybridization using a probe having the sequence of SEQ ID NO:4 or its complement is 2X SSC, 1% SDS at 50°C for about 20 to about 30 minutes.

One skilled in the art would be able to identify complete clones of OMP106 polypeptide coding sequence using approaches well known in the art. The extent of OMP106 polypeptide coding sequence contained in an isolated clone may be ascertained by sequencing the cloned insert and comparing the deduced <u>size</u> of the polypeptide encoded by the open reading frames (ORFs) with that of OMP106 polypeptide and/or by comparing the deduced amino acid <u>sequence</u> with that of known amino acid sequence of purified OMP106 polypeptide. Where a partial clone of OMP106 polypeptide coding sequence has been isolated, complete clones may be isolated by using the insert of the partial clone as hybridization probe. Alternatively, a complete OMP106 polypeptide coding sequence can be reconstructed from overlapping partial clones by splicing their inserts together.

Complete clones may be any that have ORFs with deduced amino acid sequence matching that of OMP106 polypeptide or, where the complete amino acid sequence of the latter is not available, that of a peptide fragment of OMP106 polypeptide and having a molecular weight corresponding to that of OMP106 polypeptide. Further, complete clones may be identified by the ability of their inserts, when placed in an expression vector, to produce a polypeptide that binds antibodies specific to the amino-terminal of OMP106 polypeptide and antibodies specific to the carboxyl-terminal of OMP106 polypeptide.

Nucleic acid sequences encoding OMP106-derived polypeptides may be produced by methods well known in the art. In one aspect, sequences encoding OMP106-derived polypeptides can be derived from OMP106 polypeptide coding sequences by recombinant DNA methods in view of the teachings disclosed herein. For example, the coding sequence of OMP106 polypeptide may be altered creating amino acid substitutions that will not affect the immunogenicity of the OMP106 polypeptide or which may improve its immunogenicity. Various methods may be used, including but not limited to oligonucleotide directed, site specific mutagenesis. These

and other techniques known in the art may be used to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, 1985, Science 229:1193-1210.

Further, DNA of OMP106 polypeptide coding sequences may be truncated by restriction enzyme or exonuclease digestions. Heterologous coding sequence may be added to OMP106 polypeptide coding sequence by ligation or PCR amplification. Moreover, DNA encoding the whole or a part of an OMP-derived polypeptide may be synthesized chemically or using PCR amplification based on the known or deduced amino acid sequence of OMP106 polypeptide and any desired alterations to that sequence.

The identified and isolated DNA containing OMP106 polypeptide or OMP106-derived polypeptide coding sequence can be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved DNA may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In an alternative method, the desired DNA containing OMP106 polypeptide or OMP106-derived polypeptide coding sequence may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired sequence, for example, by size fractionation, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that contain OMP106 polypeptide or OMP106-derived polypeptide coding sequence enables generation of multiple copies of such coding sequence. Thus, the coding sequence may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted coding sequence from the isolated recombinant DNA.

5.8. RECOMBINANT PRODUCTION OF OMP106 POLYPEPTIDE AND OMP106-DERIVED POLYPEPTIDES

OMP106 polypeptide and OMP106-derived polypeptides of the invention may be produced through genetic engineering techniques. In this case, they are produced by an appropriate host cell that has been transformed by DNA that codes for the polypeptide. The nucleotide sequence encoding OMP106 polypeptide or OMP106-derived polypeptides of the invention can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted polypeptide-coding sequence. The nucleotide sequences encoding OMP106 polypeptide or OMP106-derived polypeptides is inserted into the vectors in a manner that they will be expressed under appropriate conditions (e.g., in proper orientation and correct reading frame and with appropriate expression sequences, including an RNA polymerase binding sequence and a ribosomal binding sequence).

A variety of host-vector systems may be utilized to express the polypeptide-coding sequence. These include but

are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. Preferably, the host cell is a bacterium, and most preferably the bacterium is *E. coli*, *B. subtilis* or *Salmonella*.

The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In a specific embodiment, a chimeric protein comprising OMP106 polypeptide or OMP106-derived polypeptide sequence and a pre and/or pro sequence of the host cell is expressed. In other specific embodiments, a chimeric protein comprising OMP106 polypeptide or OMP106-derived polypeptide sequence and an affinity purification peptide is expressed. In further specific embodiments, a chimeric protein comprising OMP106 polypeptide or OMP106-derived polypeptide sequence and a useful immunogenic peptide or polypeptide is expressed. In preferred embodiments, OMP106-derived polypeptide expressed contains a sequence forming either an outer-surface epitope or the receptor-binding domain of native OMP106 polypeptide.

Any method known in the art for inserting DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the polypeptide coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of a nucleic acid sequence encoding OMP106 polypeptide or OMP106-derived polypeptide may be regulated by a second nucleic acid sequence so that the inserted sequence is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the inserted sequence may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of inserted sequences

include, but are not limited to the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3′ long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42) for expression in animal cells; the promoters of β-lactamase (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), *tac* (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), λP_L, or *trc* for expression in bacterial cells (see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); the nopaline synthetase promoter region or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120) for expression implant cells; promoter elements from yeast or other fungi such as the Gal4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter.

Expression vectors containing OMP106 polypeptide or OMP106-derived polypeptide coding sequences can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the inserted OMP106 polypeptide or OMP106-derived polypeptide coding sequence. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes

in the vector. For example, if the OMP106 polypeptide or OMP106-derived polypeptide coding sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of OMP106 polypeptide or OMP106-derived polypeptide in *in vitro* assay systems, *e.g.*, binding to an OMP106 ligand or receptor, or binding with anti-OMP106 antibodies of the invention, or the ability of the host cell to hemagglutinate or the ability of the cell extract to interfere with hemagglutination by *M. catarrhalis*.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As explained above, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered OMP106 polypeptide or OMP106-derived polypeptide may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to

ensure the desired modification and processing of the foreign protein expressed.

5.9. REAGENTS

The polypeptides, peptides, antibodies and nucleic acids of the invention are useful as reagents for clinical or medical diagnosis of *M. catarrhalis* infections and for scientific research on the properties of pathogenicity, virulence, and infectivity of *M. catarrhalis*, as well as host defense mechanisms. For example, DNA and RNA of the invention can be used as probes to identify the presence of *M. catarrhalis* in biological specimens by hybridization or PCR amplification. The DNA and RNA can also be used to identify other bacteria that might encode a polypeptide related to the *M. catarrhalis* OMP106.

The polypeptides and peptides of the invention may be used to prepare polyclonal and monoclonal antibodies that can be used to further purify compositions containing the polypeptides of the invention by affinity chromatography. The polypeptides and peptides can also be used in standard immunoassays to screen for the presence of antibodies to *M. catarrhalis* in a sample.

It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and processes for their preparation and use appear in the following example.

6. EXAMPLE: ISOLATION AND CHARACTERIZATION OF THE OMP106 POLYPEPTIDE AND GENE ENCODING SAME FROM STRAIN ATCC 49143 OR OTHER STRAINS

6.1. MATERIAL AND METHODS

6.1.1. HEMAGGLUTINATION ASSAY

Hemagglutination by *M. catarrhalis* was tested as described by Soto-Hernandez et al. (J. Clin. Microbiol.

27:903-908) except 5%, instead of 3%, v/v erythrocytes were used in a slide agglutination assay. Initial hemagglutination assays were performed using 20 μg of bacterial cells (wet weight). Since *M. catarrhalis* ATCC strain 49143 grown on blood agar plates at 35°C gave a strong hemagglutination reaction, it was selected as the reference strain. Serially diluting ATCC strain 49143 in 1:2 dilutions resulted in decreasing hemagglutination reactions. Scores of ++++ to + were based on the hemagglutination observed by ATCC strain 49143 after serial 1:2 dilutions so that a + reaction resulted using 1/4 the number of cells required to achieve a +++ reaction.

6.1.2. INHIBITION OF HEMAGGLUTINATION

M. catarrhalis ATCC 49143 cell suspension was serially diluted 1:2, and the dilution that yielded a + hemagglutination reaction when 7 μl of Dulbecco's phosphate buffered saline and 7 μl of 5% (v/v) human O^+ erythrocytes was used to assay inhibition of hemagglutination by simple sugars and sugar derivatives. To determine if simple sugars or sugar derivatives could inhibit hemagglutination by *M. catarrhalis*, 7 μl of a given sugar at 500 mM was mixed with 7 μl of *M. catarrhalis* cells and incubated for 5 minutes to allow the sugar to interact with the cells. Then 7 μl of 5% (v/v) human O^+ erythrocytes were added and the hemagglutination was scored after 1 minute. Each sugar and sugar derivative was tested for the ability to inhibit hemagglutination. Then the stock of each sugar and sugar derivative was serially diluted 1:2, and these dilutions were assayed for their ability to inhibit hemagglutination using the procedure described above. In this manner, the minimal concentration of carbohydrate required to inhibit hemagglutination was determined.

6.1.3. LIGAND AND RECEPTOR BINDING

M. catarrhalis binding to animal cell glycolipid receptors was examined using thin layer chromatography (TLC)

fractionation of the host cell glycolipids and labeled cell overlay of the chromatogram following the procedures described by Magnani et al., 1982, J. Biol. Chem. 257:14365-14369. Briefly, glycolipids obtained from Matreya Inc. (Pleasant Gap, PA) were resolved on high performance thin layer chromatograph plates (HPTLC) in chloroform, methanol, water (5: 4: 1) The plates were either stained with orcinol at 100°C, or were overlaid with ^{125}I-labeled *M. catarrhalis* blebs prepared as previously described (Murphy and Loeb, 1989, Microbial Pathogen. 6:159-174) at 2 X 10^6 cpm/ml for 2 hours. The plates were then washed 5 times, dried and exposed to X-ray film.

6.1.4. <u>OG EXTRACTION OF OMPS</u>

Strains of *M. catarrhalis* were each grown at 35°C at 200 rpm in 1 liter of Mueller Hinton broth in a 4 liter flask. Outer membrane protein (OMP) preparations were isolated by treating 50 mg of cells (wet weight) with 0.67 ml of 1.25% n-octyl β-D-glucopyranoside (i.e., octyl glucoside; OG) in phosphate buffered saline (PBS) for 30 minutes at room temperature. Cells were pelleted in a microcentrifuge for 5 minutes and the supernatant was used as an octyl glucoside extract. Comparison of protein profiles of these extracts from a number of strains of *M. catarrhalis* to those of blebs (i.e., outer membrane vesicles) isolated by differential centrifugation, which are highly enriched for outer membrane proteins (OMPs) from *M. catarrhalis* (Murphy and Loeb, 1989, Microbial Pathogen. 6:159-174) indicates the octyl glucoside extracts contain predominately outer membrane proteins of *M. catarrhalis* (Fig. 1). This indicated that octyl glycoside extraction provided a more rapid procedure with a higher yield of outer membrane proteins as compared to outer membrane proteins prepared from blebs.

6.1.5. <u>PROTEOLYTIC DIGESTION OF OMP106</u>

50 mg of cells from ATCC strain 49143 in 1 ml of Dulbecco's phosphate buffered saline were digested for 1 hour

at room temperature with the following proteases: TLCK-treated chymotrypsin (5 mg), Proteinase K (5 mg), TPCK-treated trypsin (5 mg), or protease V8 (100 Units). All proteases were obtained from Sigma Chemicals (St. Louis, MO). Immediately following the protease treatment, cells were washed once in PBS and resuspended in 1 ml of PBS and the hemagglutinating activity was tested. Additionally, protease-treated bacterial cells were extracted with octyl glucoside so the outer membrane proteins could be resolved to identify specific proteins that may have been digested by the proteases.

6.1.6. NON-HEMAGGLUTINATING CULTIVARS

Normally, hemagglutinating *M. catarrhalis* cultures are grown in shaker flasks containing Mueller Hinton Broth at 35 to 37°C at 200 rpm for 24 to 48 hours. Cells taken directly from a blood agar plate or an agar plate of Mueller Hinton media also express the hemagglutinating phenotype. To select for a non-hemagglutinating (NHA) cultivar, ATCC strain 49143 was serially passaged every 5 days in static cultures grown in Mueller Hinton broth at 35°C. With each passage, inoculum was taken only from the surface of the broth culture. By the second passage, a floating mat of cells had developed and this mat of cells was used as the inoculum for subsequent cultures. Serial culturing in this manner produced NHA cultivars of ATCC 49143 typically after three passages.

6.1.7. ISOLATION OF OMP106 POLYPEPTIDE

OMP106 polypeptide from outer membrane extract of *M. catarrhalis* ATCC 49143 is detected (e.g., by silver staining or anti-OMP106 antibodies) in denaturing gels only after the extract has been incubated at 100°C for five minutes. In order to determine if the appearance of the OMP106 band after incubation at 100°C is the result of lower molecular weight proteins aggregating during boiling, or if the boiling allows a normally aggregated protein to enter the

gel, an unboiled octyl glucoside outer membrane extract of ATCC 49143 was analyzed on a native polyacrylamide gel. Specific regions of the gel including that immediately below the sample well were excised and boiled. The resulting samples were then resolved on a denaturing polyacrylamide gel and stained with silver stain (Silver Stain Plus, Catalog number 161-0449, BioRad Laboratories, Richmond, CA). For N-terminal sequencing, an octyl glucoside outer membrane extract of ATCC 49143 was mixed with PAGE sample buffer containing SDS, and was incubated for 5 minutes in boiling water bath. The proteins were then resolved on a 12% PAG with SDS and transferred to a PVDF membrane by electroblotting. The region of the membrane containing the OMP106 band was then cut out for amino-terminal sequencing. None of the PAGE procedures used to isolate the OMP106 polypeptide used reducing agents in the sample or gel buffers.

6.1.8. ANTI-OMP106 ANTISERUM

Antiserum to OMP106 were prepared by resolving OMP106 polypeptide from a HA cultivar of ATCC 49143 in a denaturing sodium dodecylsulfate polyacrylamide gel as previously described (Lammeli, 1970, Nature 227:680-685), and cutting the OMP106-containing band out of the gel. The excised band was macerated and injected into a rabbit to generate antiserum to OMP106 polypeptide. The antiserum was used to inhibit hemagglutination as described in section 6.1.2. supra, but using the antiserum in place of the carbohydrate. The antiserum was also examined for complement-mediated cytotoxic activity against *M. catarrhalis* as described in section 7.

6.1.9. WESTERN BLOTS WITH ANTI-OMP106 ANTISERUM

M. catarrhalis ATCC 49143, ATCC 43628, ATCC 43627, ATCC 43618, ATCC 43617, ATCC 25240, ATCC 25238, and ATCC 8176; *M. ovis* ATCC 33078; *M. lacunata* ATCC 17967; *M. bovis* ATCC 10900; *M. osloensis* ATCC 10973; *Neisseria gonorrhoeae*

(clinical isolate); and *N. meningitidis* ATCC 13077 were grown on chocolate agar plates for 48 hours at 35°C in 5% CO_2. Cells were removed by scraping the colonies from the agar surface using a polystyrene inoculating loop. Cells were then solubilized by suspending 30 μg of cells in 150 μl of PAGE sample buffer (360 mM Tris buffer [pH 8.8], containing 4% sodium dodecylsulfate and 20% glycerol), and incubating the suspension at 100°C for 5 minutes. The solubilized cells were resolved on 12% polyacrylamide gels as per Laemmli and the separated proteins were electrophoretically transferred to PVDF membranes at 100 V for 1.5 hours as previously described (Thebaine et al. 1979, Proc. Natl. Acad. Sci. USA 76:4350-4354) except 0.05% sodium dodecylsulfate was added to the transfer buffer to facilitate the movement of proteins from the gel. The PVDF membranes were then pretreated with 25 ml of Dulbecco's phosphate buffered saline containing 0.5% sodium casein, 0.5% bovine serum albumin and 1% goat serum. All subsequent incubations were carried out using this pretreatment buffer.

PVDF membranes were incubated with 25 ml of a 1:500 dilution of preimmune rabbit serum or serum from a rabbit immunized with OMP106 polypeptide (as described above) for 1 hour at room temperature. PVDF membranes were then washed twice with wash buffer (20 mM Tris buffer [pH 7.5.] containing 150 mM sodium chloride and 0.05% Tween-20). PVDF membranes were incubated with 25 ml of a 1:5000 dilution of peroxidase-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove Penn. Catalog number 111-035-003) for 30 minutes at room temperature. PVDF membranes were then washed 4 times with wash buffer, and were developed with 3,3'diaminobenzidine tetrahydrochloride and urea peroxide as supplied by Sigma Chemical Co. (St. Louis, Mo. catalog number D-4418) for 4 minutes each.

6.1.10. ANTI-OMP106 IMMUNOFLUORESCENCE STAINING OF CELL SURFACE

M. catarrhalis ATCC 49143 was grown overnight at 35°C in a shaking water bath in Mueller Hinton broth. The cells were pelleted by centrifugation and then resuspended in an equal volume of Dulbecco's modification of phosphate buffered saline without calcium or magnesium (PBS/MC). 20 μl of the cell suspension was applied to each of 5 clean microscope slides. After setting for 10 seconds, the excess fluid was removed with a micropipettor, and the slides were allowed to air dry for 1 hour. The slides were then heat fixed over an open flame until the glass was warm to the touch. The slides were initially treated with 40 μl of 1:40 dilution of anti-OMP106 antiserum or preimmune serum from the same animal diluted in PBS/MC, or PBS/MC for 10 minutes, then washed 5 times with PBS/MC. The slides were treated with 40 μl of 5 μg/ml PBS/MC of fluorescein isothiocyanate-labeled goat antibody to rabbit IgG (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, MD catalog number 02-15-06). The slides were incubated in the dark for 10 minutes and were washed 5 times in PBS/MC. Each slide was stored covered with PBS/MC under a cover slide and was viewed with a fluorescence microscope fitted with a 489 nm filter. For each sample five fields-of-view were visually examined to evaluate the extent of straining.

6.2. RESULTS

6.2.1. HEMAGGLUTINATION ACTIVITY

The agglutination activity of *M. catarrhalis* with respect to erythrocytes is species specific with the strongest activity observed with human erythrocytes. Rabbit erythrocytes are also agglutinated by *M. catarrhalis*, but less dramatically than are human cells. The erythrocytes from mouse, horse or sheep were not agglutinated (see Table 1).

Table 1: Strength of hemagglutination of erythrocytes from various species using *M. catarrhalis* ATCC 49143

Source of erythrocytes	Score for hemagglutination[a]
Human	++++
Rabbit	++
Mouse	-
Horse	-
Sheep	-

[a] ++++ = strongest agglutination, - indicates no agglutination

6.2.2. OMP106 RECEPTORS AND LIGANDS

M. catarrhalis hemagglutination activity is due to binding to globotetrose (Gb_4). Blebs from hemagglutinating strains bind to a glycolipid having Gb_4, whereas non-hemagglutinating strains do not bind to the same glycolipid (see Fig. 2). *M. catarrhalis* hemagglutination activity is inhibited by monosaccharide constituents of Gb_4 or derivatives of such monosaccharides, with the most potent inhibitors being N-acetyl galactosamine and galactose (especially the alpha anomer of the galactose) (see Table 2).

Table 2: The minimum concentration of sugars required to inhibit hemagglutination (MIC) by *M. catarrhalis*

Sugar	MIC (mM)*
D-Glucose	>167
D-Mannose	83
D-Galactose	41
L-Fucose	83
N-acetyl-D-Glucosamine	>167
N-acetyl-D-Galactosamine	41
Methyl-α-Glucose	>167
Methyl-α-Mannose	167
Methyl-α-Galactose	10
Methyl-ß-galactose	83

* Minimal concentration of sugar required to inhibit a 1+ hemagglutination reaction by *M. catarrhalis* ATCC 49143 with 5% washed human O+ erythrocytes.

Both N-acetyl galactosamine and alpha-galactose are part of the Gb_4 tetrasaccharide. The correlation between hemagglutination and binding to Gb_4, and the observation that hemagglutination is inhibited by monosaccharides that comprise the Gb_4 receptor suggest that *M. catarrhalis* cells bind to the tetrasaccharide Gb_4. This tetrasaccharide is present on human erythrocytes and tissues, and could mediate *M. catarrhalis* attachment to eukaryotic membranes.

6.2.3. IDENTIFICATION OF OMP106 POLYPEPTIDE

Proteolytic digestion of *M catarrhalis* cells, and subsequent analysis of hemagglutination by the digested cells demonstrated that protease treatment with chymotrypsin and proteinase K destroyed the hemagglutination activity, and treatment with trypsin partially destroyed hemagglutination activity, indicating the hemagglutinating activity is protein mediated. Analysis of the OMP protein profiles of protease digested *M catarrhalis* cells showed that multiple proteins had been degraded in each sample, so the profiles did not

provide a clue as to which protein is directly responsible for or indirectly contributed to the hemagglutination activity (see Fig. 3).

Since protease treatment indicated a polypeptide is directly or indirectly responsible for hemagglutination activity, we used SDS-PAGE to compare the OMP profiles from hemagglutinating strains with the OMP profiles from non-hemagglutinating strains (Fig. 4). Analysis of the differences between these profiles indicated that the hemagglutinating strains had two unique polypeptides, one with an apparent molecular weight of 27 kD (designated OMP27) and the other was the only protein with an apparent molecular weight of greater than 106 kD (designated OMP106). Notably, the OMP106 polypeptide band was absent in the OMP preparations of various protease treated cells that have reduced or no hemagglutination activity, whereas the OMP27 band was present in the OMP preparation of proteinase K treated cells that have no hemagglutination activity. Additionally, the OMP106 polypeptide band was not degraded by proteinase V8 digestion, which did not affect hemagglutination activity of treated cells.

6.2.4. <u>OMP PROFILE OF NHA CULTIVARS</u>

Serial culturing of NHA cultivar of ATCC 49143 in static culture at 35°C produced a NHA cultivar (designated 49143-NHA) by the third passage of the culture. This loss of the hemagglutination activity was repeatable. Analysis of OMP profiles of OG outer membrane extracts of the HA and NHA cultivars showed that the OMP106 polypeptide band was missing from the 49143-NHA extract (Fig. 5). This suggested that OMP106 polypeptide is the *M. catarrhalis* hemagglutinin (i.e., OMP106 polypeptide binds Gb_4 receptor or is a subunit of a homopolymeric protein that binds Gb_4 receptor) or forms a part of the *M. catarrhalis* hemagglutinin (i.e., OMP106 polypeptide is a subunit of a heteropolymeric protein that binds Gb_4 receptor).

6.2.5. OMP106 AND HEMAGGLUTINATION

Polyclonal antiserum raised to ATCC 49143 OMP106 polypeptide neutralized hemagglutination by ATCC 49143, as well as that by heterologous ATCC 43627. This further supports the conclusion that *M. catarrhalis* hemagglutinating activity comprises OMP106 polypeptide, and that OMP106 polypeptide is antigenically conserved among strains. See also Fig. 9A, which shows antibodies in the polyclonal antiserum binding OMP106 polypeptide of heterologous *M. catarrhalis* strains.

6.2.6. OUTER SURFACE LOCATION OF OMP106

Rabbit anti-OMP106 antiserum was used in indirect immunofluorescence staining to determine if OMP106 polypeptide is exposed on the outer surface of *M. catarrhalis* cells. *M. catarrhalis* cells treated with anti-OMP106 antiserum stained more intensely and uniformly than did cells treated with preimmune serum or PBS/MC. This indicated that in intact *M. catarrhalis* cells OMP106 polypeptide was reactive with anti-OMP106 antibodies. This result indicates that OMP106 polypeptide is exposed on the outer surface of *M. catarrhalis*. This finding is consistent with OMP106 polypeptide having a role in hemagglutination and, moreover, indicates that OMP106 polypeptide would be useful as a vaccine.

6.2.7. PROPERTIES OF OMP106 POLYPEPTIDE

OMP106 polypeptide exists as a large protein complex in its native state or aggregates when extracted with octyl glucoside. This conclusion is supported by the finding that extracting *M. catarrhalis* cells with octyl glucoside will solubilize OMP106 polypeptide, but the extracted OMP106 polypeptide does not enter denaturing PAGs unless the extract is first incubated at 100°C (Fig. 6). Further, the OMP106 polypeptide band does not appear to form from lower molecular weight polypeptides that polymerize or aggregate upon

heating, since OMP106 polypeptide in a non-heat denatured sample is trapped in the sample well and enters the resolving gel only if the sample has been first incubated at 100°C. This biochemical property is very useful for identifying OMP106 polypeptide in various gels.

Using octyl glucoside extracts of *M. catarrhalis*, then incubating the extracts with sodium dodecyl sulfate at 100°C, and resolving the proteins on a denaturing polyacrylamide gel, we have estimated the apparent molecular weight of OMP106 polypeptide from various strains of *M. catarrhalis*, specifically those of ATCC 25238, ATCC 25240, ATCC 43617, ATCC 43618, ATCC 43627 and ATCC 43628, to range from about 180 kD to about 230 kD (Fig. 9A), whereas the OMP106 polypeptide of strain ATCC 49143 appears to have an apparent weight of about 190 kD (Fig. 6).

OMP106 polypeptide of strain ATCC 49143 was extracted from the gel slice and its N-terminal was sequenced. The sequencing showed the N-terminal of OMP106 polypeptide from the outer membrane of ATCC 49143 to be IGISEADGGKGGANARGDKSIAIGDIAQALGSQSIAIGDNKIV (SEQ ID NO:1). Additionally, an internal peptide of OMP106 produced by Lys-C digest (Fernandez et al., 1994, Anal Biochem 218:112-117) has been isolated and its sequence determined to be GTVLGGKK (SEQ ID NO:2).

We generated three oligonucleotide probes. Two probes correspond to the internal peptide GTVLGGKK, one has the following sequence GGNACNGTNCTNGGNGGNAARAAR (SEQ ID NO:3), the other has the following sequence GGNACNGTNTTRGGNGGNAARAAR (SEQ ID NO:7). The other probe, Mc 5-72, encoding an internal fragment (SEQ ID NO:5) of the amino-terminal sequence of OMP106 (SEQ ID NO:1) has the following sequence GAAGCGGACGGGGGGAAAGGCGGAGCCAATGCGCGCGGTGATAAATCCATTGCTATTGGTGACATTGCGCAA (SEQ ID NO:4). Hybridization of the Mc 5-72 probe to a complete HindIII or DraI digest of *M. catarrhalis* DNA in each instance produced a single band in Southern blot analysis (Fig. 7). The hybridizing band in the HindIII

digest has an approximate size of 8.0 kb; the hybridizing band in the DraI digest has an approximate size of 4.2 kb (Fig. 7).

6.2.8. CONSERVATION OF OMP106 POLYPEPTIDE

Western blot analysis of outer membrane protein extracts of a number of *M. catarrhalis* strains and related species of bacteria showed that the anti-OMP106 antibodies binds to a polypeptide of about 180 Kd to about 230 kD in many *M. catarrhalis* strains, both HA and NHA strains or cultivars (Fig. 9A). The anti-OMP106 antibodies did not bind to any polypeptide in the protein extracts of related bacteria (Fig. 8A). These results demonstrate the following: 1) Anti-OMP106 antibodies may be used to specifically identify and distinguish *M. catarrhalis* from related species of bacteria. 2) OMP106 polypeptide may be used to generate antibodies that have diagnostic application for identification of *M. catarrhalis*. 3) Antibodies to OMP106 polypeptide of one strain (e.g., OMP106 of ATCC 49143) may be used to identify and isolate the corresponding OMP106 polypeptide of other *M. catarrhalis* strains. Interestingly, the Western blot results show that many of the NHA *M. catarrhalis* strains have OMP106 polypeptide in OG extracts of their outer membranes. This finding and the fact that silver staining of OMPs from OG outer membrane extracts of NHA *M. catarrhalis* strains after PAGE does not reveal a band in the 180 kD to 230 kD range indicate that OMP106 polypeptide is expressed by most *M. catarrhalis* strains or cultivars but that, in order to be active in hemagglutination (i.e., binding to receptor on mammalian cell surfaces) or silver stainable, the OMP106 polypeptide must be appropriately modified in some manner. Apparently only HA strains and cultivars are capable of appropriately modifying OMP106 polypeptide so that it can mediate bacterial binding to hemagglutinin receptor on mammalian cell surfaces.

7. EXAMPLE: EFFICACY OF OMP106 VACCINE: CYTOTOXIC ACTIVITY OF ANTI-OMP106 ANTISERUM

Complement-mediated cytotoxic activity of anti-OMP106 antibodies was examined to determine the vaccine potential of OMP106 polypeptide. Antiserum to OMP106 polypeptide of a HA cultivar of ATCC 49143 was prepared as described in Section 6.1.8. supra. The activities of the pre-immune serum and the anti-OMP106 antiserum in mediating complement killing of *M. catarrhalis* were examined using the "Serum Bactericidal Test" described by Zollinger et al. (Immune Responses to *Neiserria meningitis*, *in* Manual of Clinical Laboratory Immunology, 3rd ed., pg 347-349), except that cells of HA and NHA *M. catarrhalis* strains or cultivars were used instead of *Neiserria meningitis* cells.

The results show that anti-OMP106 antiserum mediated complement-killing of a HA cultivar of heterologous *M. catarrhalis* ATCC 43627 but not a NHA cultivar of *M. catarrhalis* ATCC 43627 or the NHA *M. catarrhalis* ATCC 8176. Table 3 summarizes the complement mediated cytotoxic activities of pre-immune serum and anti-OMP106 antiserum against a HA cultivar of ATCC 43627.

Table 3: Complement mediated cytotoxic activities of pre-immune serum and anti-OMP106 antiserum

	Cytotoxic Titer[1]	
	Pre-immune	Anti-OMP106
Experiment 1	16	128
Experiment 2	8	64

[1] The titer is in the highest dilution at which a serum can mediate complement killing of a HA cultivar of ATCC 43627 (e.g., 16 represents a 16 fold dilution of the serum), the larger the number, the higher the cytotoxic activity or titer.

As shown in Table 3, the anti-OMP106 antiserum has 8 fold greater cytotoxic activity than the pre-immune serum.

This finding indicates that OMP106 polypeptide is useful as a vaccine against HA *M. catarrhalis* strains and cultivars.

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.